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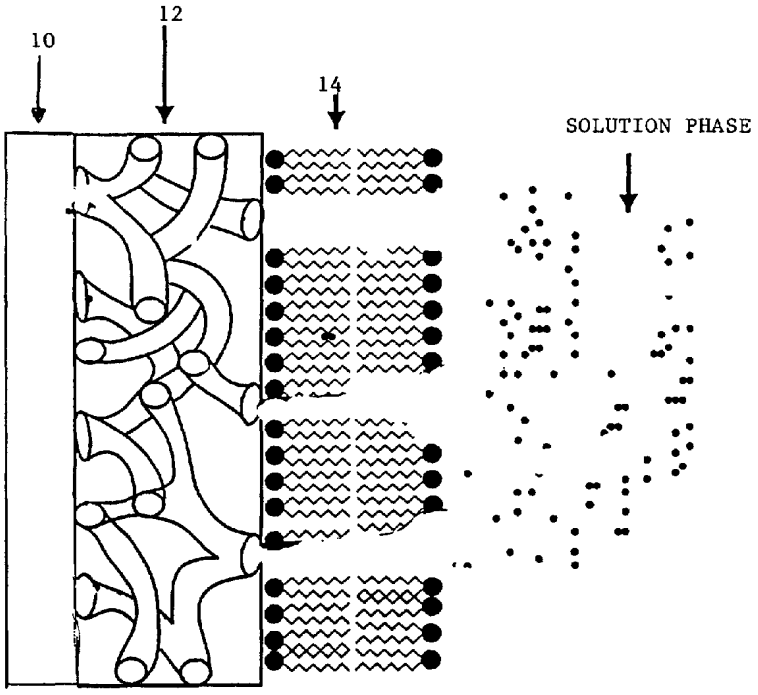
(54) Title: MEMBRANE MIMETIC ARCHITECTURES ON NANOPOROUS MATERIALS



(57) Abstract: A membrane structure including a nonoporous inorganic oxide support material (12) and a bilayer lipid membrane (14) thereon is provided.

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MEMBRANE MIMETIC ARCHITECTURES ON NANOPOROUS MATERIALS

The present invention claims the benefit of provisional patent application Ser. No. 60/198,262, filed on April 17, 2000.

FIELD OF THE INVENTION

The present invention relates to membrane mimetic architectures on nanoporous materials organized as substrate supported thin films in a full range of morphological features as dictated by the support geometry, including e.g., nanoparticles, microspheres, microtubules and the like. More particularly, the present invention relates to membrane mimetic architectures such as supported bilayers, tethered bilayers and hybrid bilayers on nanoporous materials organized as thin films onto solid supports in a variety of structural and morphological geometries. This invention was made with government support under Contract No. W-7405-ENG-36 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Bilayer lipid membranes (BLMs) are well known in the chemical and biological fields. Such membranes have been widely described for sensor applications. Early designs for such BLMs often had the bilayer spread directly upon a planar solid substrate surface such as glass or gold. This design did not allow for an ionic reservoir on both sides of the membrane and thus limited the usefulness of such structures, e.g., in studying membrane transport functions. Lang et al. (U.S. Patent No. 5,756,355) describe a bilayer lipid membrane sensor including (1) a gold recording surface, (2) a first lipid layer which is an imperfect layer of a thiolipid which includes the residue of two phospholipid molecules linked to each end of a disulphide group, each through an oxyethylene chain which is short enough to allow the thiolipid to become anchored to the gold surface by self-assembly, but long enough to trap an aqueous layer between the gold surface and the bottom of the thiolipid layer, the thiolipid being attached to the gold surface and the imperfect layer being completed by a phospholipid which provides an unattached fluid phase at room temperature, and (3) a second lipid layer of phospholipid. The membranes of Lang et al. are bound to the surface in a manner which traps a layer of water (from about 1 to 50 Angstroms, thick) between the solid surface and the lipid part of the first layer. This layer of water enables

receptor proteins (e.g., trans-membrane proteins) which extend beyond the membrane to adopt a configuration which more closely conforms to that found in nature and enables them to respond to the binding of a ligand in a correspondingly natural fashion. Raguse et al. (Langmuir, vol. 14, pp. 648-659, 1998) and Raguse et al. (U.S. Patent No. 5,798,030) describe a tethered lipid bilayer membrane structure that also provides for a liquid region between a substrate surface and the membrane.

In another supported membrane design, Galla et al. (U.S. Patent No. 5,846,814) describe a solid support, a lipid bilayer as a membrane, spacers incorporated between the soild support and the lipid bilayer and a receptor embedded in the lipid bilayer. These membrane biosensors are characterized in particular by the spacer, which consists of a molecule of ethanolamine forming an ester bond to the phosphatide group of the lipid bilayer, an oligopeptide in helical or pleated sheet structure formed from 4-20 C_2 -C_{10} -α-amino acids and a reactive group which enters into a chemical or physicochemical bond with the solid support. The spacer was designed to avoid contact of the supporting solid with any incorporated biological receptor molecule in the bilayer membrane.

Despite the progress in this area, substantial research continues in the development of membrane mimetic architectures as bilayer membranes formed on solid supports. Such systems remain attractive model systems that can mimic selected functions of biological membranes.

An object of the present invention is to provide a membrane mimetic structure utilizing a nanoporous substrate as the support.

Yet another object of the present invention is to provide a biological sensor for direct application, such a sensor including a substrate supported nanoporous thin film as a transducer element with a bilayer membrane thereon, the bilayer membrane including receptor molecules for a target biological material such as a protein toxin or the like.

SUMMARY OF THE INVENTION

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, the present invention provides a membrane structure including a substrate, a nanoporous inorganic oxide support material upon said substrate and a lipid membrane thereon said nanoporous inorganic oxide support material.

In one embodiment, the nanoporous support material is hydrophilic such that the nanoporous support material can contain an aqueous phase for the membrane to reside over.

In one embodiment, the present invention provides a substrate of a non-planar configuration for the nanoporous inorganic oxide support material and the lipid membrane.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a schematic view of a membrane structure including the nanoporous inorganic oxide support material and the lipid membrane in accordance with the present invention.

FIGURE 2 is a schematic diagram of an experimental set-up for conducting Fluorescence Recovery After Photobleaching (FRAP) measurements on a membrane architecture in accordance with the present invention.

DETAILED DESCRIPTION

The present invention concerns membrane mimetic architectures, e.g., supported bilayers, on a nanoporous thin film. More generally, the present invention concerns membrane mimetic materials composed, e.g., of single bimolecular layers, supported on the surface of hydrated nanoporous thin films in aqueous ambient phases.

In the present invention, pre-hydrated nanoporous inorganic oxide supports that display a regular and/or irregular array of water-filled nanometer scale pore-channels, provide a tenacious aqueous cushion to decouple the membrane, e.g., bilayer, architecture from local substrate chemistry and keep the membrane head-groups well-hydrated. As a result, stable single membranes, e.g., bilayers, which retain the essential membrane properties (i.e., fluidity, co-operativity, transport, recognition and the like), can be produced. Such a supported membrane structure reproduces the properties of a real cell membrane and can be used in fundamental studies of cellular processes as well as used in sensor applications. Nanoporous inorganic oxide support materials are sometimes referred to as mesoporous support materials.

In one embodiment, the nanoporous support material is mesoporous in that it provides a dense array of pore-openings (regular or otherwise) over macroscopic distances at the surface of the said support material.

The present invention provides the following advantages over present approaches to membrane mimetic architectures. First, the nanoporous support material can provide water-filled channels that are in direct contact with the hydrophilic head groups of a membrane, e.g., the bilayer membrane, so that integral membrane proteins may reside in the membrane without loss of activity. In effect, the intra-cellular loops of the trans-membrane protein are allowed to exist in their natural state thereby preserving the function of the protein (e.g., structural changes of the intra-cellular loops via signaling). A comparable technology that preserves the intra-cellular environment for integral membrane proteins is the tethered bilayer structures of Raguse et al. (U.S. Pat. No. 5,798,030).

The water filled channels in the nanoporous support material can be replenished. They allow the creation and maintenance of differential ionic strengths and potentials across the bilayer membrane. Other membrane mimetic architectures do not typically allow this.

The structure of the present invention can be used to study many important issues in biology. These include, e.g., cell-signaling events, the function of integral membrane proteins, endocytosis (the translocation of biomolecules across a membrane and into a cell), protein-membrane and protein-receptor interactions and the like. The fact that one can access both sides of a membrane with molecules and with characterization tools is critical for the study of such fundamental aspects of cell phenomena.

In addition to fundamental studies of cellular biology, the structure of the present invention may have applications in chemical and biological sensors, in solar energy conversion, and the like. For example, the structure of the present invention can be used in a biosensor as a "dry" sensor for biological molecules. As the nanoporous support can be exposed to air without drying out the channels and, therefore, without destroying the membrane, this structure could function as a biosensor in air. This application can have applications in bio-threat reduction efforts. Another sensor application could be in enzymatic sensors that involve integral membrane proteins and where enzymatic action is triggered by a binding event. Finally, the structure of the present invention could be used in electrochemical or impedance sensor applications where a recognition event triggers the opening of an ion channel or a pore. This structure simplifies electrochemical or impedance sensing as there is no need for a water cushion below the bilayer as in the gated ion

channel biosensor of Raguse et al. In effect, the nanoporous support material can be directly deposited upon a conductive layer and the lipid membrane bilayer added on top of the nanoporous support material. In this structure, the conductive layer would provide the conducting film needed in impedance or electrochemical sensing.

By the term "liposome" is meant a bilayer structure including a natural or synthetic phospholipid membrane or membranes, and optionally other membrane components such as cholesterol and protein. By the term "leaflet" is meant a single layer of phospholipid in a bilayer of a liposome or membrane structure.

In accordance with the present invention, a membrane structure is provided and includes a nanoporous support material having pores in the range of from about 2 nm to about 50 nm in size, preferably from about 2 nm to about 20 nm in size, more preferably from about 3 nm to about 10 nm in size. Other pore sizes may be used if desired and can be controlled in the preparation of the nanoporous material. The nanoporous support material is formed upon a substrate that can be in a variety of geometries, e.g., in the form of a sheet, of nanoparticles, of microspheres, of microtubules and the like. The surface of the nanoporous material can be hydrophilic or hydrophobic although it will be hydrophilic when prepared by use of an UV/ozone treatment in accordance with the preferred practice of the present invention. Hydrophilic nanoporous materials are preferred as they would result in an aqueous phase being retained within the pores providing the necessary water to the membranes of the present invention.

In one embodiment, the nanoporous material can be hydrophobic and a silane material such as octadecyltrichlorosilane (OTS) is applied onto the hydrophobic surface of the nanoporous material. Thereafter, a hybrid bilayer membrane is formed by application of vesicle fusion methods.

The head group region of the membranes can be selected from the group consisting of groups normally associated with naturally occurring or synthetic lipids such as glycerol, phosphatidyl choline, phosphatidyl ethanolamine, mono-, di- or tri-methylated phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, disubstituted head groups as found in cardiolipins, ganglioside head groups,

sphingomyelin head groups, plasmalogen head groups, glycosyl, galactosyl, digalactosyl, sulfosugar, phosphosugar, N-acetyl neuramic acid, sialic acid, aminosugar head groups, carbohydrate head groups, gal(beta1-3)galNAc(beta1-4)›NAcNeu(alpha2-3!gal(beta1-4)glc-ceramide, oligomers of ethylene glycol, ethylene glycol, oligomers of propylene glycol, propylene glycol, amino acids, oligomers of amino acids, combinations of oligomers of ethylene glycol or propylene glyco functionalised with amino acids or other ionic species or any combination or derivative of the above.

In a preferred embodiment, the head group is a phosphatidyl choline group.

An important part of the preparation of the nanoporous support is that the initial surfactant concentration is less than the critical micelle concentration, allowing accessibility to a continuous range of silica/surfactant/solvent phase space and allowing control of the final film structure. This control allows preparation of interfacially-organized cubic phase as well as lamellar and hexagonal structures. Additionally, the pore size can be controlled to approximately 4 nm or less to larger pore sizes up to 20 nm. The surfactant can be cationic, anionic, or nonionic. The surfactant used in the Examples, Brij56 from Aldrich Chemical Co., is non-ionic. Other surfactants that can be used include cationic surfactants such as alkyammonium salts, gemini surfactants, cetylethylpiperidinium salts, and dialkyldimethylammonium, anionic surfactants such as sulfates, sulfonates, phosphates, and carboxylic acids, and nonionic surfactants (with the hydrophilic group not charged) such as primary amines, poly(oxyethylene oxides), octaethylene glycol monodecyl ether and octaethylene glycol monohexadecyl ether.

Although silica sols are discussed primarily here, other metal oxides can be used in the preparation of the nanoporous supports of the present invention. In particular, along with silicon, titanium, zirconium, aluminum, and combinations thereof, can be used in the preparation of these nanoporous supports.

The structure of the liposomes may be as a multilamellar vesicle (MLV), a small unilamellar vesicle (SUV), a large unilamellar vesicle (LUV), and the like, although SUVs and LUVs are generally preferred as they can provide a single bilayer upon the nanoporous substrate.

The membranes, e.g., supported bilayers, on nanoporous support materials of the present invention have a number of applications. In one embodiment of the invention shown in Fig. 1,

the supported bilayer **14** can be on a nanoporous inorganic oxide film **12** on a substrate support **10**. Such a substrate support can be a solid such as glass, metals (e.g., gold, silver, platinum and the like), conducting oxides (e.g., indium-tin oxide or ITO), semi-conductors, e.g., silicon, zinc-selenide and the like, polymers or plastics. Such articles, i.e., the supported bilayer on a nanoporous film on a substrate support have use in fundamental studies of biophysics and have use in chemical and biological sensors. The substrates can also be in other non-planar configurations. The ability to support membrane spanning molecules (integral membrane proteins) in an environment where the intracellular loops are not constrained by close proximity to the solid support (as they are with supported bilayers) is a distinct advantage. Other prior membrane architectures that provide this are polymer cushions (see, Spinke et al., Biophys. J., vol. 63, pp. 1667-1671, 1992) or tethered bilayers (see, Raguse et al., Langmuir, vol. 14, pp. 648-659, 1998). The other advantage is that chemical or biochemical molecules can be brought up to either side or these membranes in order to test, e.g., for enzyme activity of a trans-membrane protein. The membranes supported on nanoporous films could serve as membrane-based chemical and biological sensors. For example, the ability to support the nanoporous films on metals (e.g., silver, gold, platinum, palladium and the like) would be ideal for use in electrochemical sensing whether it is via ion channels and impedance measurements (e.g., a variant on the ion channel gated biosensor approach of Raguse et al.) or enzymes and conductivity measurements. Where the support is an optical transducer (e.g., fiber optic or waveguide), such membrane architectures could be used for fluorescence or absorption based transduction.

In another embodiment of the invention, the supported bilayer can be on substrates in the form of nanoporous particles. Such nanoporous particles can be hydrophilic and contain water within the pores whereby a bilayer membrane can be maintained upon the particle surface.

Membrane based sensor approaches could be employed where the active membrane layer is protected by the nanoporous support. As the nanoporous support can be extremely hydrophilic, it will, in effect, never dry out and can therefore be exposed to air. As the channels in the nanoporous support will retain water, they could serve to carry biological molecules (e.g., protein toxins) to the membrane triggering tranduction. Proximity-based fluorescence changes

could be used in transduction as well as other optical changes induced by binding. Currently, there is no biological sensor that can be used directly in air to monitor biomolecules. One possible application would be in environmental detection of biological agents.

The present invention is more particularly described in the following examples which are intended as illustrative only, since numerous modifications and variations will be apparent to those skilled in the art.

EXAMPLE 1

Amphiphile-templated organic-inorganic hybrid films were deposited upon clean oxidized silicon (with native overlayer) or glass substrates using the procedure of Brinker et al. described in U.S. Patent No. 5,858,457. Briefly, precursor solution was prepared by addition of a non-ionic surfactant ($C_{16}H_{33}(OCH_2CH_2)_{10}OH$, available as Brij56 from Aldrich Chemical Co.) to a polymeric silica sol solution in (C_o) quantities corresponding to 1.5-5.0 weight percent (wt %), below the critical micellar concentrations (C_{mc}). The silica sol in turn was prepared by refluxing a mixture of 56.9 grams (g) of tetraethyl ortho silicate (TEOS, $Si(OC_2H_5)_4$), 48.1 g of ethanol (C_2H_5OH), 0.44 g of water, and 0.51 mg of 0.07 N hydrochloric acid at 60°C. During the addition of the surfactant, the water, ethanol and hydrochloric acid concentrations were adjusted to yield a final reactant mixture in the mole ratios of: 1 TEOS: 22 C_2H_5OH: 5 H_2O: 0.004 HCl: 0.054-0.18 surfactant. Films were deposited onto freshly oxidized silicon with native oxide overlayer (SiO_2/Si). Both dip-coating and spin-coating procedures were used. Dip-coated samples were prepared by withdrawing the substrates from the precursor solution at about 4-40 centimeters per minute (cm/min). Spin-coated samples were achieved by spinning the substrates at about 3000 revolutions per minute (rpm) under about 1 milliliter (ml) of the precursor sol mixture for 120 seconds (s). After formation of the inorganic-organic structure, the organic template phase was removed by the exposure of the as-formed, substrate supported surfactant-silicate nanostructured thin films to ultraviolet (UV) light, 184-257 nm range, produced by a low pressure Hg discharge gridlamp in quartz envelope, maintained in a closed chamber under laboratory ambient conditions for from about 30 minutes to about 90 minutes. The nanostructures of the resultant substrate-supported thin films were verified using a combination of powder X-ray diffraction, transmission electron microscopy, and Fourier transform infrared spectroscopy. Each analytical

technique established that the films were structurally consistent with those reported earlier by, e.g., Brinker et al.

EXAMPLE 2

Large unilamellar vesicles of egg phosphatidyl choline (egg-PC) combined with low concentrations (1 mole percent) of a fluorescent probe, N-(Texas Red Sulfonyl)-1,2-dihexadeconyl-sn-glycero-3-phosphoethanloamine, triethylammonium salt (Texas Red DHPE), were used to form a supported bilayer on the nanoporous support from Example 1. Appropriate amounts of egg-PC and Texas Red were mixed in chloroform and then dried under argon, followed by vacuum for at least 4 hours. The mixture was then reconstituted in a phosphate buffered saline (PBS) solution at a pH of 7.4 to produce 1 ml of solution. Suspension of the lipids was achieved by vortex mixing. Large unilamellar vesicles were then produced by extruding the vesicle solution through a 0.1 micrometers (μm) polycarbonate filter using a mini-extruder (available from Avanti-Polar Lipids, Inc.)

A bilayer membrane on the nanoporous support was then formed from the large unilamellar vescile solution by placing about 50 microliters (μl) drops of the vesicle solution on a Teflon® sheet which was placed inside a plastic petrie dish. The nanoporous support was then placed over the drop and allowed to incubate for about 1 hour. After incubation, the petrie dish was then filled with PBS solution. Excess lipids and fluorophores were then removed by rinsing the sample with copious amounts of PBS solution.

The resultant bilayer membrane on a nanoporous support was then characterized using fluorescence imaging. Fluorescence excitation of the Texas Red fluorophore was accomplished using the 514 nanometer (nm) line of a Spectra-Physics argon ion laser. Fluorescence emitted from fluorophores incorporated in the membrane was imaged using a Zeiss Axiovert 135 inverted microscope equipped with a 530 nm long pass filter to remove the excitation laser and a Cohu 4910 thermoelectric (TE)-cooled CCD Camera. Wide field imaging using a 20-times objective was used to determine the homogeneity of the supported bilayer membrane. A typical wide field image of a bilayer membrane on nanoporous supports showed a uniform and largely defect-free surface of the supported bilayer membrane.

Lateral fluidity of the fluorophores incorporated in the membrane was also determined by performing fluorescence recovery after photobleaching experiments. Using a high intensity laser, a small area of the membrane was photobleached. The recovery of this photobleached area was then imaged as a function of time after photobleaching. The guassian intensity profile of the photobleached area at each time interval was used to determine the diffusion constant. A plot of the full-width half-maximum of each profile as a function of time yielded a line whose slope was directly related to the diffusion constant of the fluorophore. The measured diffusion constants for a bilayer membrane on a silicon-supported nanoporous film, along with bilayers on various additional substrates are shown in Table 1.

TABLE 1. Measured Diffusion Constants for Texas Red in Supported Bilayer Membranes

Substrate	Diffusion Constant (mm^2/sec)
Glass	1.45
Silicon	3.83
OTS on Silicon	5.07
Nanoporous film on Silicon	1.55

Although the present invention has been described with reference to specific details, it is not intended that such details should be regarded as limitations upon the scope of the invention, except as and to the extent that they are included in the accompanying claims.

WHAT IS CLAIMED IS:

Claim 1. A membrane structure comprising a substrate, a nanoporous inorganic oxide support material upon said substrate, and a lipid membrane thereon said nanoporous inorganic oxide support material.

Claim 2. The membrane structure of claim 1 wherein said nanoporous inorganic oxide support material contains water within the pores.

Claim 3. The membrane structure of claim 1 wherein said support material is characterized as hydrophilic.

Claim 4. The membrane structure of claim 1 wherein said support material is characterized as having pores within the range of from about 2 nm to about 20 nm.

Claim 5. The membrane structure of claim 2 wherein said support material is characterized as having pores within the range of from about 2 nm to about 20 nm.

Claim 6. The membrane structure of claim 1 wherein said substrate is a material selected from the group consisting of conducting, semi-conducting or insulating materials.

Claim 7. The membrane structure of claim 2 wherein said substrate is a material selected from the group consisting of conducting, semi-conducting or insulating materials.

Claim 8. The membrane structure of claim 1 wherein said substrate is characterized as a non-planar configuration.

Claim 9. The membrane structure of claim 2 wherein said substrate is characterized as a non-planar configuration.

Claim 10. The membrane structure of claim 2 wherein said lipid membrane is characterized as a hybrid bilayer.

Claim 11. The membrane structure of claim 2 wherein said lipid membrane is characterized as a tethered bilayer lipid membrane.

Claim 12. The membrane structure of claim 2 wherein said lipid membrane is characterized as a supported bilayer lipid membrane.

Claim 13. The membrane structure of claim 1 wherein said substrate is a conductive material.

Claim 14. The membrane structure of claim 2 wherein said substrate is a conductive material.

Claim 15. The membrane structure of claim 13 wherein said conductive material is metallic.

Claim 16. The membrane structure of claim 14 wherein said conductive material is metallic.

Claim 17. A membrane structure comprising a substrate, a nanoporous inorganic oxide support material upon said substrate, a silane layer directly thereon said nanoporous inorganic oxide support material, and a lipid membrane layer thereon said silane layer.

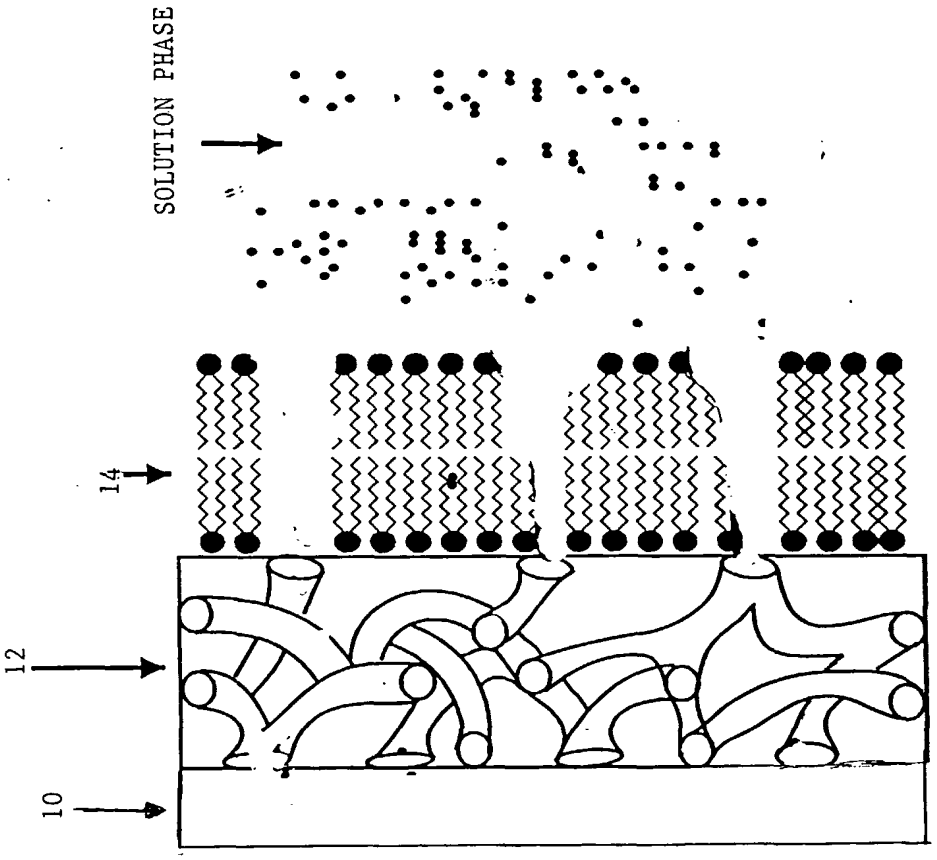

Fig 1

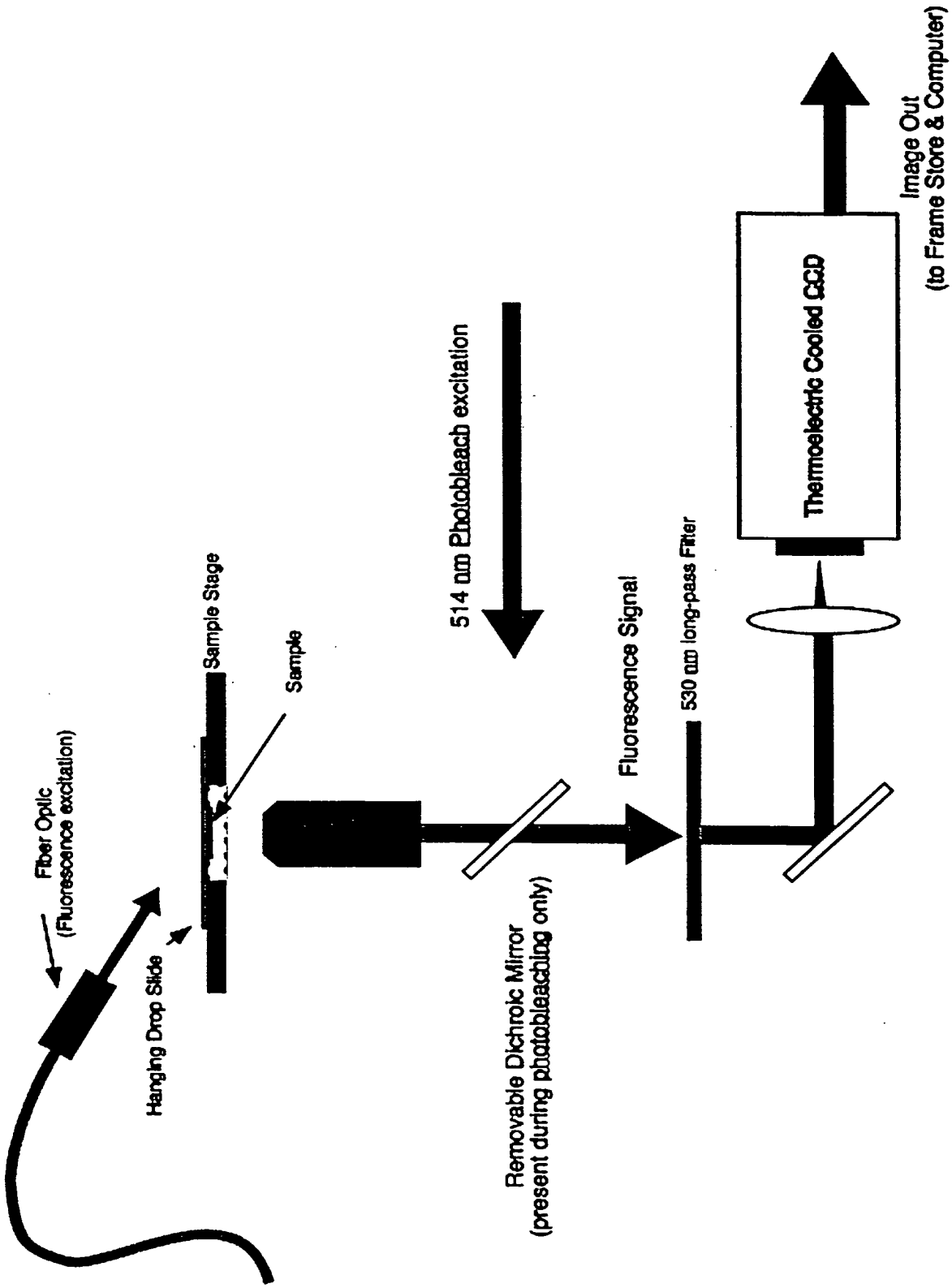
Fiber Optic
(Fluorescence excitation)
Hanging Drop Slide
Sample Stage
Sample
514 nm Photobleach excitation
Removable Dichroic Mirror
(present during photobleaching only)
Fluorescence Signal
530 nm long-pass Filter
Thermoelectric Cooled CCD
Image Out
(to Frame Store & Computer)

Fig 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/12253

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : B32B 3/06
US CL : 428/307.3, 307.4, 319.1; 204/403.08; 435/289.1, 817

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 428/307.3, 307.4, 319.1; 204/403.08; 435/289.1, 817

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,111,221 A (FARE et al) 05 May 1992 (05.05.1992), the whole document. column 5, lines 27-28.	1, 3, 6, 8, 13, 15 ---------- 2, 4, 5, 7, 9-12, 14, 16, 17
Y	US 5,756,355 A (LANG et al) 26 May 1998 (26.05.1998), column 5, lines 22-32.	2
Y	US 4,490,216 A (MCCONNELL et al) 25 December 1984 (25.12.1984), figures 1-3, column 9, lines 35-37.	17
Y	US 5,846,814 A (GALLA et al) 08 December 1998 (08.12.1998), column 4, lines 54-58.	17
A	US 5,798,030 A (RAGUSE et al) 25 August 1998, the whole document.	1-17

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2002 (16.06.2002)	18 JUL 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Hai Vo Telephone No. (703) 308-0661

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